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Molecular characterization of *Turnip yellows virus* - a new pathogen of mustards in Serbia

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Summary: In 2018, a total of 58 mustard samples from two different localities in Serbia (Rimski Šančevi and Senta) were collected and tested for the presence of *Turnip yellow virus* (TuYV), *Cauliflower mosaic virus* (CaMV) and *Turnip mosaic virus* (TuMV) by DAS-ELISA. TuYV was serologically detected in 42 tested samples while all collected samples were negative for CaMV and TuMV. By using aphid transmission tests, five test plants were inoculated with two ELISA-positive TuYV samples of naturally infected mustard plants. Virus species identification was performed by amplification of a 780 bp fragment in all tested samples using the specific primers TuYVorf0F/TuYVorf0R. The RT-PCR products from two isolates 88Sal (MK144816) and 98Bni (MK144817) were sequenced and compared with the GenBank sequences of TuYV. Serbian isolates showed the highest identity with Poland TuYV isolate (EU022489). Phylogenetic analysis showed that TuYV isolates from Serbia were clustered with other TuYV sequences retrieved from the GenBank.
Key words: DAS-ELISA, mustards, phylogenetic analysis, RT-PCR, *Turnip yellows virus*

Introduction

Mustard plant species belong to the genus *Brassica* and *Sinapis* of the Brassicaceae family. The most common species are pale yellow or white mustard (*Sinapis alba*) and black or brown mustard (*Brassica nigra*). Mustard plant originates from the region around the Mediterranean Sea and the Middle East. Now it can be found all over the world as cultivated species and weeds. It is well known for its condimental, therapeutic and flavouring properties (Sharma et al., 2018). Mustard leaves are regarded as a vegetable, while the seeds can be used as a condiment and constitute the source of mustard oil (Manohar et al., 2009).

A number of viruses such as *Beet western yellow virus* (BWYV), *Cauliflower mosaic virus* (CaMV), and *Turnip mosaic virus* (TuMV) can infect Brassica crops (Farzadfar et al., 2007). BWYV was originally detected in the USA in the late 1950s and it was reported that it caused different symptoms in a number plant species, such as stunting and chlorosis, and it also resulted in yield losses in sugar beet, spinach, turnip, and lettuce (Duffus, 1961; Duffus, 1977). The International Committee for the Taxonomy of Viruses (ICTV) has re-classified many of the isolates previously named as BWYV, which have not been proved to infect sugar beet (*Beta vulgaris* L.), as an independent species within the genus *Polerovirus*, as *Turnip yellows virus* (TuYV) (Mayo, 2002).

TuYV causes harmful diseases in many species of Brassica and other crops worldwide, including economically important vegetable, oilseed, forage, tobacco and ornamental crops (Jay et al., 1999; Sharma et al., 2013; Wang et al., 2015; Farzadfar et al., 2007). The wide range of cultivated plants, as well as a variety of weed species susceptible to TuYV, extend the range of potential reservoir hosts where the virus can successfully overwinter and provide an inoculum source for future viral infectious (Latham et al., 2003). TuYV is transmitted by a few aphid species in a persistent manner, with the green-peach aphid, *Myzus persicae* as the main vector (Hauser et al., 2002).

Considering that TuYV is one of the most important and widespread viruses in brassica crops worldwide, the virus presence in oilseed rape crop in Serbia (Milošević et al., 2015), as well as the common

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presence of a lot of aphids known as virus vectors, TuYV potentially represents a limiting factor for a successful production of mustard plants in Serbia. Moreover, virus-like symptoms are increasingly noticed in the production of mustards in Serbia. The main objectives of this study were to identify the virus presence in other Brassica species in Serbia and to study their taxonomic position based on sequencing of P0 gene.

Material and Methods

Sample collection

In 2018, mustard leaf samples showing virus-like symptoms, including mild reddening of leaf margins followed by mild yellowing leaves, were collected from two crops at two different localities: Rimski Šančevi (South Bačka District) and Senta (North Banat District) in Serbia. A total of 33 symptomatic *Sinapis alba* plants (10 samples from Rimski Šančevi and 23 from Senta locality) and 25 *Brassica nigra* plants (10 samples from Rimski Šančevi and 15 from Senta locality) were collected. The samples were transported in plastic bags, and stored at 4°C until ELISA test was performed or at -20°C until RNA extraction and RT-PCR analysis were performed.

Serological detection

All 58 collected samples were tested using double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) according to manufacturer's instructions (Loewe Biochemica, Germany) specific for detection of three most frequent viruses on Brassica species: *Turnip yellow virus* (TuYV), *Cauliflower mosaic virus* (CaMV) and *Turnip mosaic virus* (TuMV) (Nooh, 2012). Fresh leaves of plant samples were ground in extraction buffer (1:10 wt/vol). The commercial positive and negative controls (as well as the extracts from the healthy mustard leaf tissue) for the above-mentioned viruses, were included in each ELISA test. After incubation with p-nitrophenyl phosphate (Sigma-Aldrich, USA), absorbance values were determined at 405 nm (A405) by an ELISA microplate reader (Multiscan Ascent, Finland). Samples were considered positive if the average optical density (OD) was twice higher than the average OD of the negative control.

Aphid transmission

Myzus persicae (Sulzer) was used as a vector in aphid transmission test of the selected TuYV isolates from both localities. Nymphs were allowed to feed on the leaves of the two selected ELISA positive samples (88Sal and 98Bni), one sample per each host, for 24 h (Acquisition access period, AAP). Two groups of 6-8 aphids were thereafter transferred onto ten plants of each *Beta vulgaris, Physalis floridana, Brassica napus* 'Zlatna', *S. alba* and *B. nigra* for an inoculation access period (IAP) of 4-day. The plants and aphids were put into small cages, placed in acclimatized room with a 16 h photoperiod at 22°C. After this inoculation access period, aphids were removed. After 5 weeks, the presence of virus in inoculated plants was assessed using DAS-ELISA, with commercial kit for TuYV.

Molecular detection and sequence analysis

Four selected samples were analysed by conventional Reverse Transcription Polymerase Chain Reaction (RT-PCR) to confirm the DAS-ELISA results.

Table 1. Sequences of *Turnip yellows virus*, *Beet western yellows virus*, *Beet chlorosis virus*, *Cereal yellow dwarf virus* and *Cucurbit aphid-borne yellows virus* isolates used in the phylogenetic analysis

Virus	Isolate name	Country	Host plant	GenBank Acc. No.
TuYV	**88Sal**	**Serbia**	***Sinapis alba***	**MK144816**
TuYV	**98Bni**	**Serbia**	***Brasica nigra***	**MK144817**
TuYV	119-TuYV	Serbia	*Oilseed rape*	KU351664
TuYV	114-TuYV	Serbia	*Oilseed rape*	KR351306
TuYV	FL1	France	*Lactuca sativa*	NC003743
TuYV	TuYV-BN5	Germany	*Oilseed rape*	AF168606
TuYV	TuYV-GB	England	*Oilseed rape*	AF168608
TuYV	TuYV-FL1	France	*Lactuca sativa*	X13063
BChV	BChV-CR	USA	*Sugar beet*	AF352025
BChV	BChV-GW	California	*Sugar beet*	AF167485
BChV	BChV-2a	England	-	NC002766
CYDV-RPS	NY	-	-	NC004751
CYDV- RPV	CYDV-RPS	-	-	NC002198
BWYV	USA	USA	*Sugar beet*	AF473561
BWYV	BJ-B	China	*Sugar beet*	HM804472
BWYV	BJ-A	China	*Sugar beet*	HM804471
BWYV	USA	-	-	NC004756
CabYV	N	France	Cucurbit	X76931
CabYV	Xinjiang	China	*Cucumis melo*	EU636992
CabYV	Beijing	China	Cucurbit	EU000535
CabYV	N	France	-	NC003688

Total RNA was isolated using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) from 100 mg of freeze-dried leaves of symptomatic plants. RT-PCR was carried out using the Qiagen One-Step RT-PCR Kit with specific TuYV primers, TuYVorf0F/TuYVorf0R (Schubert et al., 1998) which flank P0 gene. The Serbian isolate of TuYV of oilseed rape (GenBank Accession No. KR351306) was used as positive control, while a tissue sample of healthy mustard leaf served as negative control. The RT-PCR reaction mixture included 400 µM each of the four dNTPs, 1 µl of RT-PCR enzyme mix, 0.6 µM each primer, and 1 µl extracted RNA in a final volume of 25 µl. Amplifications were carried out in an Eppendorf Mastercycler Gradient (Eppendorf, Germany) under the following program: 30 min at 50°C for reverse transcription, 15 min at 95°C for initial PCR denaturation step, followed by 35 cycles of 30 s at 94°C for denaturation, 30 s at 55°C for annealing, 60 s at 72°C for primer extension and 10 min at 72°C for final extension.

The amplified fragments were analysed using electrophoresis on 1% agarose gel containing ethidium bromide (0.5g/mL) and visualized using UV transilluminator. The sizes of the amplified fragments were estimated by comparison with FastRuler DNA Ladder, Low Range (Fermentas, Lithuania).

The amplified products from two selected isolates 88Sal from *S. alba* and 98Bni from *B. nigra* were sequenced in both directions (Macrogen, Korea), with primers used for its detection. The nucleotide sequences of the isolates were deposited in GenBank database (http://www.ncbi.nlm.nih.gov) and were compared with each other by calculating nucleotide (nt) and deduced amino acid (aa) identities, as well as with sequences deposited in the GenBank, using the ClustalW program (Thompson et al. 1994) and MEGA5 software (Tamura et al. 2011). A p-distance model was applied for nucleotide (nt) and deduced amino acid (aa) sequence analyses.

The phylogenetic tree was constructed using maximum parsimony method implemented in MEGA 5.0 based on two P0 gene sequences of TuYV obtained in this study, and sequences of TuYV, *Beet western yellows virus* (BWYV), *Beet chlorosis virus* (BChV), *Cereal yellow dwarf virus* (CYDV) and *Cucurbit aphid-borne yellows virus* (CabYV) isolates available in the GenBank. Genetic diversity intra and inter group of host and geographical origin were calculated with Kimura 2-parameter (K2+G) which was chosen as the best-fitting model of nt substitution.

Results and Discussion

Prior to our study, there were no data on the prevalence and diversity of viruses in mustard grown in Serbia. In this study, the presence of TuYV was studied in two *Brassica* species, *Brassica nigra* and *Sinapis alba*.

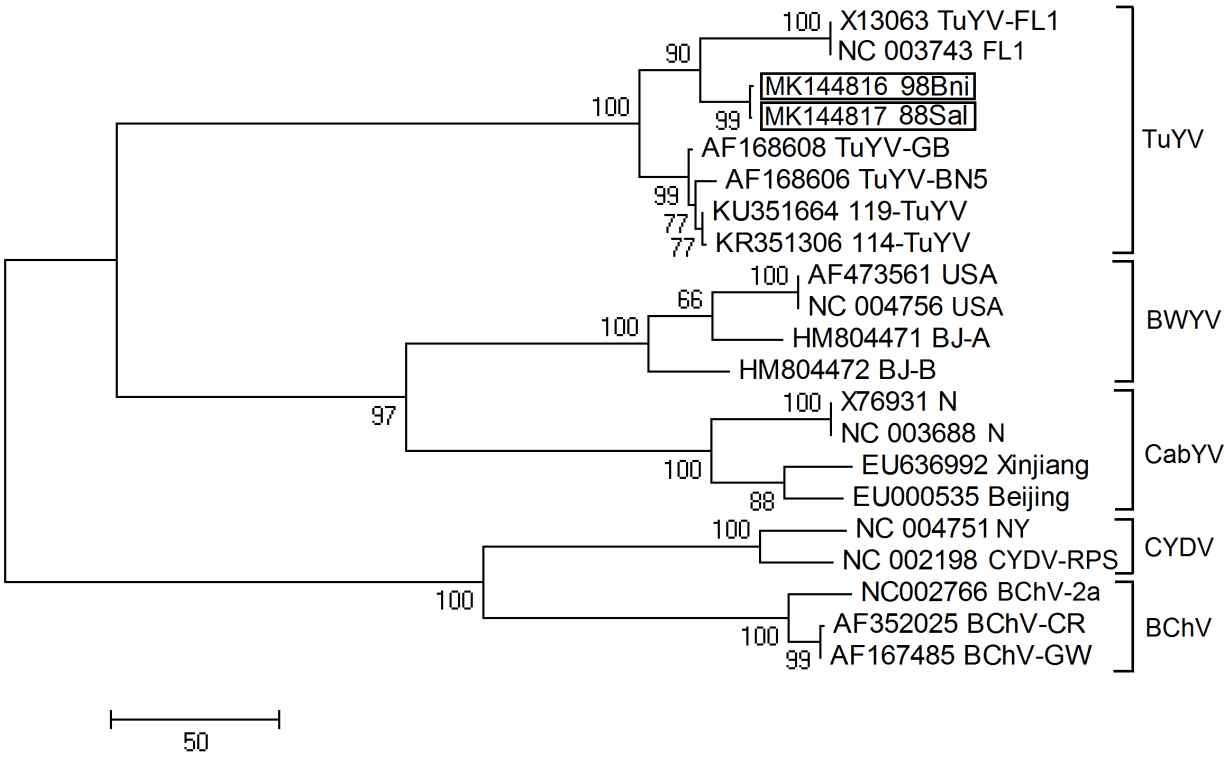

Figure 1. Maximum parsimony tree based on partial sequences of P0 gene of *Turnip yellows virus*, *Beet western yellows virus*, *Beet chlorosis virus*, *Cereal yellow dwarf virus* and *Cucurbit aphid-borne yellows virus* isolates. Phylogram was generated with MEGA5 using bootstrap analysis with 1000 replicates and bootstrap values (>50%) are shown next to relevant branches. The *Turnip yellows virus* isolates from this study are framed.

Serological detection and symptomatology in the field

During the observation of mustard fields in two localities in 2018, similar symptoms were noticed in both surveyed localities and disease incidence was estimated at 30% and 50%, respectively. Mild reddening of leaf margins followed by mild yellowing leaves were the most frequent symptoms.

Serological analysis of mustard samples showed the presence of TuYV in two localities in Serbia (Rimski Šančevi and Senta). TuYV was detected in 42 (72.4%) of the tested samples collected during the survey and tested by DAS-ELISA. CaMV and TuMV were not found in the analysed mustard samples.

The presence of TuYV on *S. alba* was higher in Senta where 78.3% samples tested positive, compared to its presence in Rimski Šančevi where 50% samples tested positive. Presence of TuYV on *B. nigra* showed similar results. The presence of TuYV on *B. nigra* was identified on 86.7% tested samples collected in Senta, while its presence in Rimski Šančevi was detected in 60% tested samples.

In some symptomatic samples, the analysed viruses were not discovered. It is more likely that other viruses infect brassica plants in Serbia, as previously reported (Farzadfar et al., 2005a; Farzadfar et al., 2005b; Shahraeen et al., 2003). Using the same method during 1998-2000, the frequency of occurrence of six viruses was determined in *B. nigra* collected from five coastal sites in Dorset (Thurston et al., 2001). Our study showed a higher frequency of TuYV in *B. nigra* compared to *B. oleracea* (43%) reported by Raybould et al. (1999). The abundance and movement of the aphid vectors, mainly *Myzus persicae* and *Brevicoryne brassicae* can cause the spread of TuYV in different surveyed regions (Afshariazad, 2001; Sohani et al., 2002). This study reports the natural occurrence of TuYV on *B. nigra* and *S. alba* in Serbia for the first time.

Aphid transmission

After about 5 weeks post-inoculation, all inoculated *P. floridana* plants manifested a very mild interveinal chlorosis, while all inoculated *B. napus* 'Zlatna' plants showed mild leaf reddening. In addition, the virus was successfully transmitted to *S. alba* and *B. nigra* that reacted with a mild reddening of leaf margins and yellowing. No symptoms were observed in *Beta vulgaris* plants. All inoculated plants of each species (except plants of *B. vulgaris*) were tested positive for TuYV.

Based on previous studies, *B. vulgaris, P. floridana*, and *B. napus* could be used as test plants in order to make distinction *Beet mild yellowing virus* (BMYV) from BWYV European isolates (reclassified as TuYV) (Hauser et al., 2000). The results of host range studies showed that Serbian TuYV isolates could infect different Brassica species, but not *B. vulgaris*. These results correspond with those previously reported by other researchers (Stevens et al., 1994; Hauser et al., 2000).

Molecular detection and sequence identity analyses

The TuYVorf0F and TuYVorf0R primers specifically amplified target cDNA fragments of 780 bp predict size and successfully detected the presence of TuYV in all four ELISA-positive samples as well as in positive control. No reaction was recorded in healthy mustard control.

The identities of the obtained products from selected isolates 88Sal and 98Bni were directly sequenced in both directions, after purification. The sequences of *S. alba* and *B. nigra* virus isolate in this study were submitted to the GenBank (MK144816 and MK144817, respectively). Sequence analysis of the of P0 gene revealed high nt identity of 99.8% (100% aa identity) among Serbian TuYV isolates from mustard. The Serbian isolates from mustard (88Sal and 98Bni) showed the highest nucleotide identity (98.9% and 99%, respectively) and amino acid identity (100%) with a Poland TuYV isolate (EU022489). As indicated by King et al. (2011), the species demarcation criteria for the genus *Polerovirus* include differences in amino acid sequence identity of any gene product greater than 10%, nucleotide identities showed that Serbian isolates belong to TuYV.

A Maximum parsimony tree (Fig. 1), reconstructed using the partial sequences of the P0 gene, showed that the TuYV isolates from this study and 19 representative isolates of previously characterized viruses within *Polerovirus* genus (TuYV, BWYV, CabYV, CYDV, and BChV) from GenBank, were clustered into five groups. Genetic diversity among five groups of isolates ranged from 0.476±0.037 to 2.514±1.122, whereas within each group and subgroup the genetic diversity was: 0.087±0.009 (TuYV), 0.095±0.011 (BWYV), 0.108±0.012 (CabYV), 0.093±0.013 (CYDV), and 0.037±0.007 (BChV). The Serbian TuYV isolates 88Sal and 98Bni were clustered within the cluster with other TuYV isolates from the GenBank and were distinct from the BWYV isolates.

On the basis of high attack on other species of the family Brassicaceae, numerous weeds in growing regions throughout the world, and distinct yield losses determined in field experiments, the control of the infestation of Brassicaceae family by TuYV is deemed necessary. Further research is needed to detect the span of this virus and inoculum sources in nature.

Conclusions

To the best of our knowledge, this is the first report of the TuYV presence on *Sinapis alba* and *Brassica nigra* in Serbia. TuYV presence was confirmed by DAS-ELISA tests and RT-PCR with specific primers. It is also the first report that confirmed the association of the symptoms of leaf yellowing and reddening and the TuYV in these species. Considering the rapid growth of mustard production in Serbia, the occurrence of TuYV

could prevent its successful production. As TuYV can be found on various crops and easily transmitted in a non-persistent manner by aphids and a wide range of hosts, continuous monitoring of TuYV status and its presence in Serbia is necessary.

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Molekularna karakterizacija Turnip yellows virus - novog patogena slačice u Srbiji

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Sažetak: : Tokom 2018. godine, sa dva lokaliteta gajenja slačice, ukupno je sakupljeno 58 uzoraka koji su serološki testirani na prisustvo *Turnip yellow virus* (TuYV), *Cauliflower mosaic virus* (CaMV) i *Turnip mosaic virus* (TuMV) korišćenjem komercijalno dostupnih kitova za DAS-ELISA test. Prisustvo TuYV serološki je dokazano u 42 sakupljena uzorka slačice, dok prisustvo CaMV i TuMV nije dokazano ni u jednom od testiranih uzoraka. Za dalja istraživanja odabrana su dva izolata TuYV prirodno zaraženih biljaka slačice, koji su uspešno preneti vašima na pet različitih test biljaka, čime je potvrđena infektivna priroda oboljenja. Molekularna detekcija obavljena je amplifikacijom fragmenta dužine 780 bp kod četiri ispitivana izolata korišćenjem specifičnih prajmera TuYVorf0F i TuYVorf0R. RT-PCR produkti izolata 88Sal i 98Bni su sekvencirani (MK144816 i MK144817) i upoređeni sa TuYV sekvencama dostupnim u GenBank bazi podataka. Izolati iz Srbije su pokazali najviši stepen nukleotidne sličnosti od 98,9% i 99% (100% aminokiselinska sličnost) sa izolatom TuYV Br iz Poljske (EU022489). Filogenetska analiza pokazala je grupisanje TuYV izolata iz Srbije zajedno sa ostalim TuYV izolatima iz GenBank baze podataka.
Ključne reči: DAS-ELISA, filogenetska analiza, RT-PCR, slačica, *Turnip yellows virus*

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